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Review

Promiscuity and specificity in BMP receptor activation

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ABSTRACT

Bone Morphogenetic Proteins (BMPs), together with Transforming Growth Factor (TGF)-β and Activins/Inhibins constitute the TGF-β superfamily of ligands. This superfamily is formed by more than 30 structurally related secreted proteins. Since TGF-β members act as morphogens, either a strict relation between a particular ligand to a distinct cellular receptor and/or temporospatial expression patterns of ligands and receptors is expected. Instead, only a limited number of receptors exist implicating promiscuous interactions of ligands and receptors. Furthermore, in complex tissues a multitude of different ligands can be found, which signal via overlapping subsets of receptors. This raises the intriguing question how concerted interactions of different ligands and receptors generate highly specific cellular signals, which are required during development and tissue homeostasis.

1. Complexity of an organism correlates with cytokine superfamily size

For their development, maintenance and survival multicellular organisms require constant intercellular communication to regulate all aspects of cellular life such as differentiation, proliferation, migration or apoptosis. No matter whether these are paracrine, endocrine or eventually autocrine stimuli the regulatory mechanisms although not exclusively but very frequently involve protein–protein interactions between a cell surface-located transmembrane receptor and a protein hormone also termed ligand. As nature seems to recycle "successful" protein scaffolds/structures and to develop new functionalities by duplicating genes rather than inventing de novo structures for each function, ligands and their receptors often form superfamilies with the number of members rising with increasing complexity of the organism [1] (the degree of complexity can be correlated with the number of different specialized cell types [2,3]). The Transforming Growth Factor (TGF)-β superfamily is no exception to this observation, with five potential ligand members in worm (*Caenorhabditis elegans*) [4], seven ligands in fly (*Drosophila melanogaster*) [5], 12 in fish (*Danio rerio*), 16 in amphibia (*Xenopus laevis*) and more than 30 ligand members in mammals (see online resources in [6]). Consistent with the hypothesis above, it seems that ligands having essential functions during early development have orthologs in all of the aforementioned phyla. For instance, orthologs of BMP-4, which is essential during early embryonic development of vertebrates [7], can be found in fly (Decapentaplegic, Dpp), in zebrafish (zBMP-4) as well as in amphibia (xBMP-4) sharing similar functions and mechanisms for dorsoventral patterning [8]. Only *C. elegans* does not seem to have a direct ortholog of the mammalian BMP-4, the genes encoding for the four ligand members, of which *daf7*, *dbl1*, and *tig2* are mapped to the human TGF-β ligands GDF-11, BMP-5 and BMP-8. The two factors Daf-7 and Dbl-1 are involved in the so-called Dauer larval development pathway regulating the body size of the larvae based on environmental conditions [4]. The function of orphan worm TGF-β member Tig-2 is unclear and Unc-129 mutant animals have been described to exhibit defects in axon outgrowth [9,10]. Thus the ligands present in worm seem to have functionalities other than patterning as is observed in other invertebrates and vertebrates [10]. Other TGF-β members have likely evolved later and exhibit functions restricted to higher organisms; e.g. GDF-9/BMP-15, which are involved in ovarian follicle development [11], the Anti-Muellerian Hormone (AMH), which plays an important role in male sex differentiation [12].

2. The TGF-β superfamily comprises four subfamilies

On the basis of their biological functions and phylogenetic analyses the more than 30 TGF-β ligands in mammals can be arranged in four main subfamilies (Fig. 1A). The Activin/Inhibin subfamily has initially been identified to regulate the expression of the pituitary Follicle-Stimulating Hormone (FSH) in the gonads ([13] for

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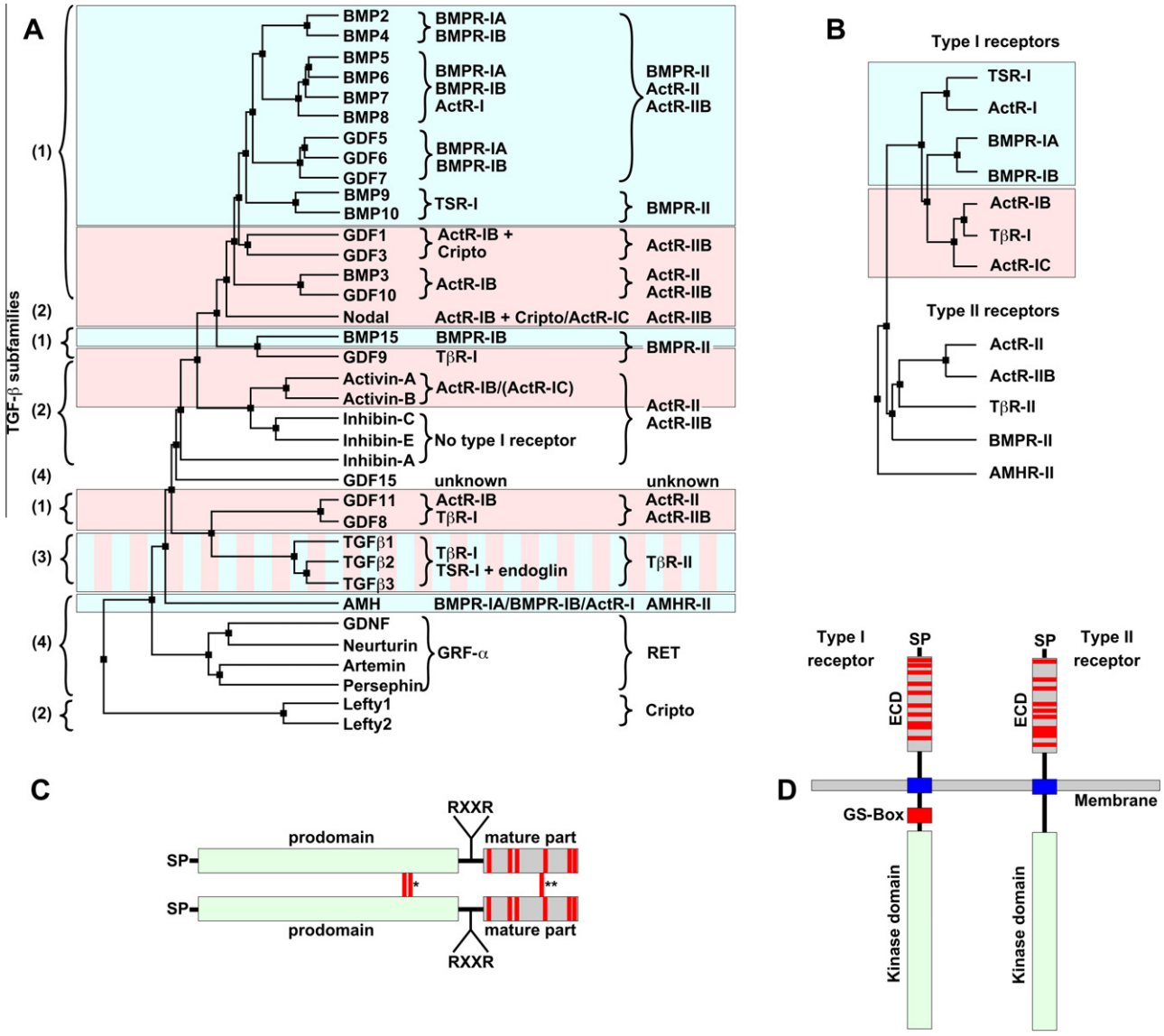

Fig. 1. (A) Phylogenetic analysis of the TGF-β ligand superfamily (only the mature region was used) showing the existence of four subfamilies as indicated on the left: BMP/GDF (1), Activin/Inhibin/Nodal (2), TGF-βs (3) and others (4). Type I and type II receptor usage is indicated next to each ligand and deduced from biophysical interaction or in vitro pulldown and crosslinking analyses. Light-blue shaded boxes emphasize SMAD 1/5/8, whereas light-red shaded boxes highlight SMAD 2/3 downstream signaling. (B) Phylogenetic tree (kinase domains were used for analysis) of TGF-β receptors highlighting type I and type II subgroup classification. Color usage for type I receptors indicates the SMAD pathway utilized (as in A). (C) TGF-β ligands are synthesized as dimeric proproteins including an N-terminal signal peptide (SP), a large prodomain and a C-terminal mature part with a characteristic cystine-knot motif. Cysteine residues are illustrated by red bars. Proteolytic processing by furin proteases occurs at the RXXR motif. Indicated by the single asterisk are the two intermolecular disulfide bonds, which are exclusive to TGF-β1/2/3 and covalently link the prodomain dimer. The intermolecular disulfide bond in the dimer of the mature part is marked by two asterisks (Lefty-1, -2, GDF-3, GDF-9 and BMP-15 lack this disulfide bond). (D) The architecture of TGF-β type I and type II receptor architecture consists of an N-terminal extracellular ligand-binding domain (EC), a single span-transmembrane and an intracellular kinase domain. The extracellular parts comprise ten cysteines in both receptor subtypes, but with a distinct sequential arrangement. An intracellular glycine/serine-rich domain (GS-box) characteristic for type I receptors is essential for kinase and downstream SMAD pathway activation.

recent review see [14]). However outside the gonads, Activins have additional functions, e.g. mesoderm induction, which is important for early body pattern determination and organogenesis (for review [15]) or in inflammation and immunity (for review [16]) (Fig. 1A). The subfamily members Nodal together with the more distant factors Lefty-1/-2 (that act as inhibitor to Nodal) are required for establishing left-right asymmetry [17] and Nodal itself is possibly also involved in maintaining embryonic stem (ES) cells in an undifferentiated state [18]. The subfamily of the TGF-β factors is the likewise smallest with only three members in mammals: TGF-β1, TGF-β2 and TGF-β3 (Fig. 1A). The TGF-βs are pleiotropic factors controlling proliferation and differentiation of many different cell types, thus TGF-β functions have been implicated in the control of immunity (e.g. by inducing FoxP3-positive regulatory T-cells a.k.a. iTregs) [19], in wound healing (e.g. promoting fibrosis through induction of extracellular matrix synthesis in different tissues and organs) [20] or for embryonic development [21]. However, best known is their dual role in the development and progression of cancer. Normally, TGF-β inhibits growth of most cell types including epithelial, endothelial and hematopoietic cells by blocking the cell cycle in the G1 phase thereby acting as tumor suppressor. But as a potent inducer of the epithelial-to-mesenchymal transition required for TGF-β's function in wound sealing, it enables carcinoma cells to spread and metastasize into normal

tissue at later stages of cancer progression [22]. Despite high sequence identities between the three TGF-β isoforms isoform-specific functions have been described [23].

The largest TGF-β subfamily is the group of Bone Morphogenetic Proteins (BMPs) and Growth and Differentiation Factors (GDFs), which comprises more than 15 ligands in human (Fig. 1A). Based on sequence similarities and their diversity in functions this subfamily can be further subdivided into the BMP-2/-4, the BMP-5/-6/-7/-8, the GDF-5/-6/-7, the GDF-8/-11, the BMP-9(GDF-2)/BMP-10, the GDF-1/-3 and the GDF-10/BMP-3 subgroups. Although the name suggests that all BMP members of the subfamily can induce bone growth – BMP-2 and BMP-7 have indeed been identified through their osteoinductive properties – comparative analysis showed that the osteoinductive capabilities of individual factors vary in potency as well as to which cell type is susceptible [24]. From gene inactivation studies in mice it becomes obvious that BMP and GDF members have various other functions (for review [25]). *Bmp2* knockout mice die due to amnion/chorion defects showing that BMP-2 is an important factor for heart development [26]. *Bmp4* deficient mice show mesoderm defects in early gastrulation [7], but BMP-4 is also involved in development of various organs, in limb patterning and skeletogenesis [27,28]. Deletion of *Bmp7* shows that it is critical for kidney development, but it also plays an important role in skeletogenesis, eye and heart development and neurogenesis [29–31]. Other members, e.g. BMP-3, BMP-5, BMP-6, BMP-8, GDF-5/-6/-7, GDF-8, GDF-10, GDF-11 could be genetically removed without causing lethality pointing towards functional redundancy in skeletal, heart and limb development [32–37]. Some BMP/GDF ligands exhibit rather specific biological functions. For instance, deletion of *Gdf8* leads to a massive increase in skeletal muscle mass [38]. Mutations occurring in cattle [39] and in human [40,41] confirm that GDF-8 is a negative regulator of muscle growth. Changes in expression level in cardiomyocytes after infarct suggest that GDF-8 is not only involved in muscle growth during development but also exhibits a regenerative function throughout life [42]. Interestingly, GDF-11, which shares extremely high amino acid sequence similarity with GDF-8 (98% similarity and 90% identity between mature human GDF-8 and GDF-11), reveals a completely distinct function in vivo. Deletion of *Gdf11* in mice resulted in an increased number of vertebral segments [43] and muscle-specific conditional deletion of *Gdf11* in mice shows that GDF-11 despite being almost identical to GDF-8 in sequence does not regulate muscle growth in vivo [44]. These unexpected large functional differences between these highly similar TGF-β members are likely due to non-overlapping temperospatial expression differences as recombinant GDF-11 can act as a negative regulator of muscle growth by inhibiting differentiation of mesenchymal cells into myogenic lineages [45]. But even for BMP subgroups with overlapping functions such as BMP-5/-6/-7 individual members of this subgroup might have developed unique functions in vivo. Besides shared functions with BMP-5 and BMP-7, BMP-6 is uniquely involved in iron hemostasis as its mRNA expression is regulated in response to the iron level. As BMP-6 itself does furthermore stimulate the expression of Hepcidin, a key regulator of iron absorption, loss of BMP-6 signaling leads to iron overload as seen in the disease hemochromatosis [46].

The fourth and most heterogeneous subfamily comprises the distant members of the TGF-β superfamily (Fig. 1A). Based on sequence similarity and/or functional properties, GDF-9, BMP-15, GDF-15 (also known as MIC-1, PLAB, TGF-PL, PDF or PTGFB), Anti-Muellerian Hormone (AMH), Glial-Derived Neurotrophic Factor (GDNF) and the related factors Artemin, Neurturin and Persephin fall outside the above described three subfamilies without, however, forming an own subfamily with a defined relationship. BMP-15 and GDF-9 are involved in follicle development. In contrast to GDF-9, BMP-15 is also expressed outside the ovary possibly indicating additional BMP-15 specific functions [47]. GDF-15 seems unique showing very low sequence similarity to all other TGF-β members (Fig. 1A). It was identified by its inhibitory activity on macrophages arguing for an anti-inflammatory function [48]. It is also strongly expressed in liver, kidney and exocrine glands [49], but even though GDF-15 expression has been investigated in various diseases [50,51], biochemical data on GDF-15 is still sparse [52]. Interestingly, GDF-15 also has neurotrophic properties (similar to GDNF). It is expressed in different brain areas and promotes survival of aminergic neurons under stress and may thus be a potential factor for the treatment of Parkinson's disease [53]. GDNF together with Artemin, Persephin and Neurturin forms an own subgroup and with the latter being the most distant TGF-β subfamily with respect to sequence homology and functions (for review [54]) (Fig. 1A). GDNF family members are neurotrophic factors for dopaminergic neurons, which regulate neurite growth, cell size as well as dopamine uptake. In vitro these factors promote survival of motoneurons and peripheral neurons. The distant relationship between the GDNF subgroup and the remaining TGF-β members also manifests in the usage of a different receptor family (see below, Fig. 1B), with GDNF and its related factors binding to a set of glycosylphosphatidylinositol (GPI)-anchored membrane proteins and signaling via the receptor tyrosine kinase receptor Ret [55].

3. Involvement of the prodomain in TGF-β ligand activation

Architecture and sequence motifs indicate that TGF-β ligands derive from a common ancestor. All members are synthesized as large dimeric proproteins containing a signal-peptide for secretion and a consensus sequence a so-called cystine-knot motif in the C-terminus (Figs. 1C, 2A,B). GDNF-related factors differ with respect to the size of the proprotein part (less than 76 residue length), which is much smaller compared to other TGF-β members ($\geqslant$250 residues). The functional significance of the prodomain might differ for the individual TGF-β members. It has been claimed that the prodomain is required for proper folding and dimerization of the TGF-β ligands, however recombinant expression of various TGF-βs, BMPs, GDFs and Activins in bacteria and subsequent refolding of the mature part in vitro suggest that the propart is not generally required for folding (e.g. [56]). For the TGF-βs the prodomain (also termed LAP for latency associated peptide) is an important regulator of factor activation being non-covalently and tightly attached to the mature part after furin cleavage. The prodomain confers latency to the TGF-βs and TGF-β activity requires active removal of the prodomain (Fig. 2C). The latter is occurring via a complex process at the cell surface involving binding of the proprotein to extracellular matrix components, LAP binding proteins such as α_v integrins and LTBP (Latent TGF-β Binding Protein) to finally strip off the prodomain by tensile forces [57]. Recent structure analysis of latent TGF-β1 now provides insight how latency is generated [58]. The interaction of the prodomain-mature part resembles a "straitjacket" with the prodomain covering all receptor epitopes of TGF-β1 thereby blocking receptor binding (Fig. 2A,B). Only few other TGF-βs are also latent, e.g. GDF-8 and -11 [59,60]. Others like BMP-4, -7, -9, -10 or GDF-5 remain associated with their prodomains after secretion, but do no exhibit a similar latency effect as TGF-βs [61]. The different biological function of the prodomain might be explained by the amino acid sequence variability, which is much larger in the prodomains than in the mature part of TGF-βs. In TGF-β1/2/3 the prodomain itself forms an obligate disulfide-linked dimer (Figs. 1B, 2A), which likely attaches to the mature region with higher affinity due to avidity effects.

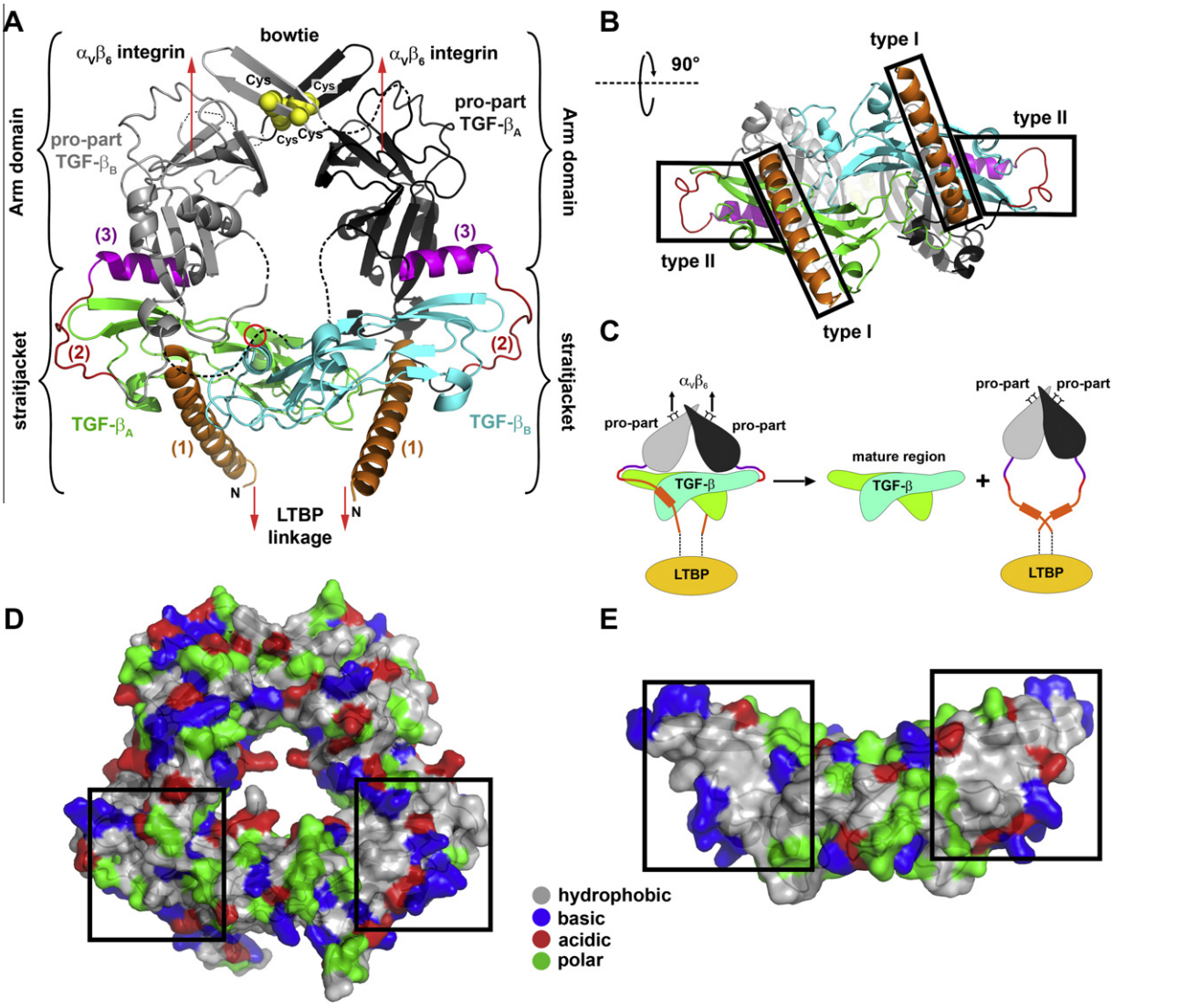

Fig. 2. (A) Ribbon representation of the TGF-β1 pro-protein. The mature part of TGF-β1 is highlighted in green and cyan; the pro-region can be separated into the Arm domain and the "straitjacket". The Arm domain comprises binding sites for $_{v}\beta_{6}$ integrins required for activation of the TGF-βs. In case of the TGF-βs, the "bowtie", which is important for dimer formation of the pro-part, contains four cysteine residues involved in intermolecular disulfide bonds stabilizing the pro-part dimer. The "straitjacket" blocks ligand–receptor interaction by blocking access to the type I receptor epitope through the N-terminal helix (1, marked in orange) as well as to the type II receptor site through the so-called latency lasso (2, marked in red). For other TGF-β ligands the helix 2 (3, indicated in magenta) blocks binding of type II receptors to the ligand. (B) As in (A) but rotated around the x-axis by 90° to highlight blocking of type I and type II receptor binding sites (indicated by boxes). (C) Release of the mature TGF-β ligand from the pro-protein complex is realized by tensile forces through binding of the N-terminus of pro-TGF-β1 to LTBP (Latent TGF-β binding protein) and interaction of Vβ6 integrins with the RGD-motif in the Arm domain. (D) For TGF-β ligands the pro-protein might also exhibit an elevated solubility under physiological conditions by shielding the hydrophobic receptor epitopes (indicated by boxes). (E) The mature region of TGF-β1 shows large, solvent accessible (conserved) hydrophobic patches (indicated by boxes), which coincide with the wrist and knuckle epitopes of BMPs.

The cysteines forming these interchain disulfide bonds are lacking or positioned differently in other TGF-β members. In addition the region involved in prodomain dimerization shows very little sequence conservation arguing for a less stable dimer formation of the prodomain [58]. Hence the prodomains of other TGF-β ligands than TGF-β1/2/3 likely bind as "monomers" and can thus be more easily displaced. A common prodomain feature, however, might be the solubility enhancement through the formation of a non-covalent prodomain-mature TGF-β complex. Biochemical studies have shown that the mature regions of TGF-β ligands alone exhibit poor solubility under physiological conditions due to large hydrophobic surface patches, whereas the proprotein complex is sufficiently soluble to allow transport by diffusion and thus might be required for other than paracrine activities (Fig. 2D,E) [62].

4. Cystine-knot architecture of TGF-β members

The key feature of the mature part of all TGF-β members is the 10-membered cystine-knot, which consists of four cysteine residues with the spacing Cys_2-X-Gly-X-Cys_3 and Cys_5-X-Cys_6 forming a ring structure (Fig. 3A–C). A third disulfide bond formed between the first and the fourth cysteine penetrates the ring structure thereby tying the knot (Fig. 3C). The cystine-knot architecture is not limited to the TGF-β superfamily and is also found in other growth factor families, but the consensus motif for TGF-β members is characterized by a stop codon one amino acid after the last cysteine residue [63]. The restriction to only one amino acid following the last cysteine residue is easily explained by the structures of the TGF-β ligands, which show a butterfly-shaped dimer arrangement with the C-termini locked up in-between the dimer interface.

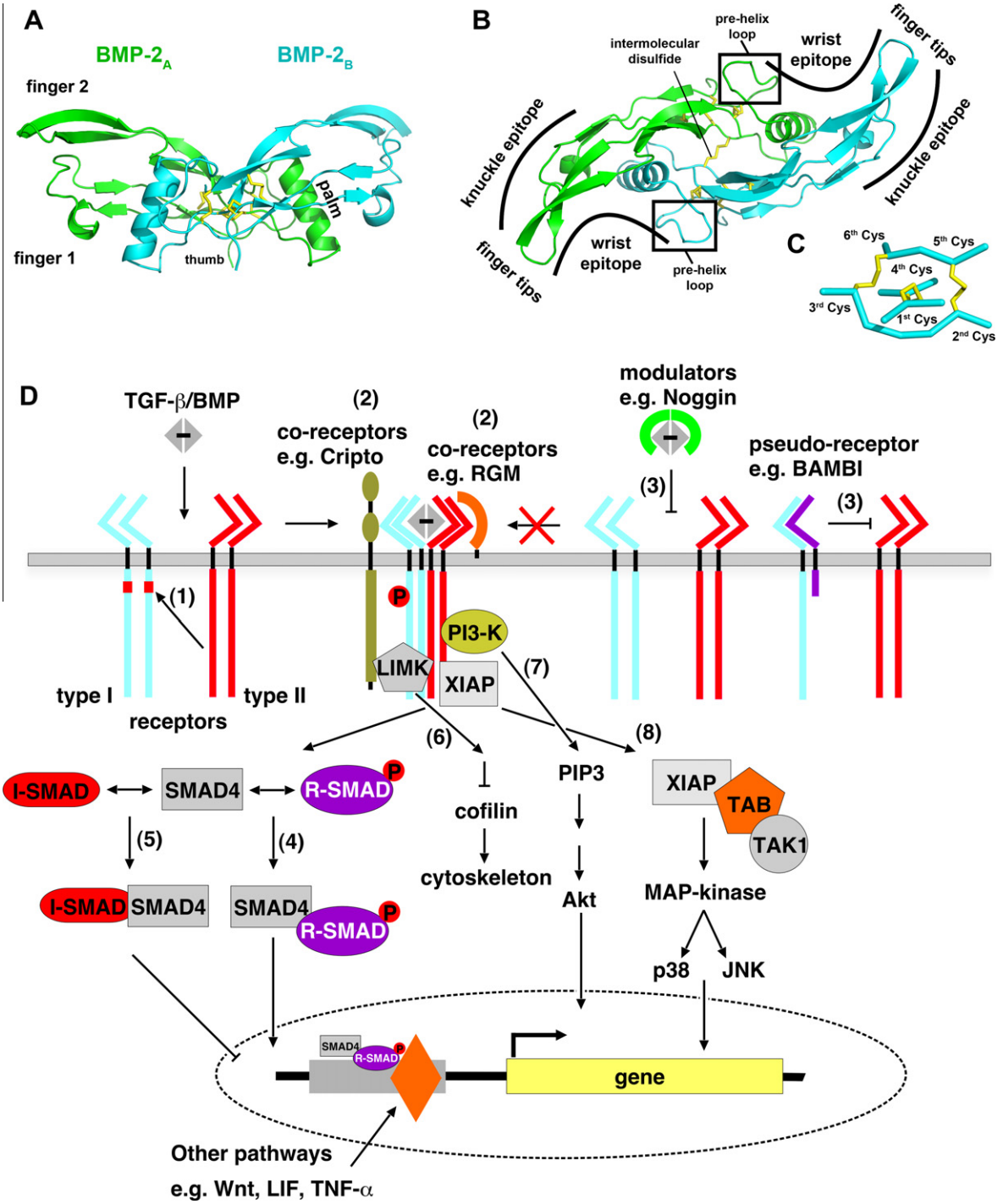

Fig. 3. (A) Ribbon representation of BMP-2. The butterfly-shaped architecture of the dimeric ligand resembles two hands assembled palm-to-palm, with the β-sheets representing fingers 1 and 2, the -helix and the dimer interface forming the palm, and the N-terminus described as thumb. The convex side of the fingers termed knuckle epitope is the binding interface for type II receptors in the BMP/GDF and Activin subgroups, TGF-βs bind their type II receptors at the finger tips. Type I receptor binding occurs in the so-called wrist epitope formed by the concave side of the fingers and the palm of the ligand. (B) As in (A) but viewed along the ligand's two-fold symmetry axis. (C) Close-up of the cystine-knot, which is formed by two disulfide bridges between Cys_2-Cys_5 and Cys_3-Cys_6 building a ring-like structure, and a third disulfide bond between Cys_1 and Cys_4 penetrating this ring. (D) Schematic representation of the TGF-β/BMP signaling cascade. Upon binding of the TGF-β ligand to its type I and type II receptors (1), the type II receptor transphosphorylates the type I receptor at the GS-box (marked by a red square), thereby activating the type I receptor kinase. Transmembrane or membrane-associated co-receptors (2) can modulate the ligand–receptor specificity or enhance binding affinity of individual TGF-β receptors. Modulator proteins (3) or pseudo-receptors (3) can impede receptor activation by either blocking the ligand or the receptors. The classical SMAD pathway is activated by phosphorylation of R-SMAD factors by the type I receptor kinase leading to complex formation between the R-SMADs and the Co-SMAD SMAD4 (4). The latter complex can translocate into the nucleus and (together with other transcription factors possibly activated by other signaling pathways) regulate gene transcription. So-called inhibitory or I-SMADs can interfere with SMAD signaling by forming inactive complexes with SMAD4 (5). Besides the canonical SMAD pathway, other signaling cascades (either SMAD independent or connected to SMAD proteins at different points) have been described for TGF-β/BMP signaling. For instance, LIMK kinases have been shown to associate with BMP receptors (or being regulated by TGF-β receptors), thereby modulating actin polymerization (6). The PI3 kinase was described to interact directly with TGF-β receptors, thereby relating TGF-β and Akt signaling (7). The anti-apoptotic protein XIAP also binds to TGF-β receptors resulting in the recruitment of the TGF-β-activated protein kinase TAK1 (8). This complex leads to activation of the MAP kinase pathway (p38 and JNK kinases), which have been shown to encode for apoptosis signals by TGF-β/BMPs.

Adding further residues to the C-terminus would disrupt this dimer architecture and thus explains the observation that adding peptide sequence tags to the C-termini usually inactivates TGF-β ligands. Most TGF-β ligands (except Lefty-1/-2, GDF-3, GDF-9 and BMP-15) have a Cys residue just ahead of the fourth cysteine of the cystine-knot, which forms an intermolecular disulfide bond stabilizing the ligand dimer (Figs. 1C, 3A–C). Although the TGF-β ligand members are considered homodimeric, the similarity in amino acid sequence and architecture has led to the proposal of heterodimeric TGF-β ligands with unique functions. Despite such heterodimers, e.g. BMP-2/6 or BMP-4/7 can be produced recombinantly in prokaryotic and eukaryotic expression systems [64,65], evidence for the in vivo existence of BMP heterodimers is so far only available for zebrafish [66] or fly [67,68], but not for mammals [69]. One possible reason against arbitrary heterodimer formation might be the sequence variability in the regions responsible for prodomain dimerization. In contrast to the high sequence similarity seen in the mature region this region (called "bowtie" in [58], see Fig. 2A) displays high variability and thus may limit or destabilize heterodimer formation.

5. TGF-β receptor assembly and downstream signaling

TGF- ligands transduce their signal via binding to two different subtypes of transmembrane serine-/threonine kinase receptors termed type I and type II (Fig. 1B,D). Although the two receptor subtypes also show differences in extracellular ligand-binding domains [5], the presence of a membrane-proximal glycine/serine-rich segment called GS-box present only in type I receptors is used for classification (Fig. 1D). Due to their nature as single span transmembrane receptors it is assumed that mechanistically ligand-induced assembly of type I and type II receptors is the trigger for downstream signaling (Fig. 3D). As TGF-β ligands are dimers, two receptors of either subtype assemble in a heterotetrameric receptor complex. Besides the classical ligand-induced receptor-oligomerization also ligand-induced activation of preformed receptor assemblies was described, with the two mechanism potentially activating different signaling cascades [70]. Subsequent phosphorylation of the type I receptor kinase by the type II receptor kinase leads to activation of the former, which then can phosphorylate and activate SMAD proteins. SMAD proteins are transcription factors that upon phosphorylation hetero-oligomerize, translocate into the nucleus and together with transcriptional co-activators or co-repressors regulate transcription of responsive genes. There are two sets of SMAD proteins (also termed R-SMAD for receptor-regulated SMAD) that interact and become phosphorylated by the type I receptors (Figs. 1A,B and 3D). Structural and functional studies have revealed that a cytoplasmic loop segment (called L45 loop) close to the GS-box of the type I receptor and a corresponding loop segment in the SMAD protein (called L3 loop) determines the specificity, which set of SMAD proteins are substrates for the respective type I receptor kinase [71,72]. The SMAD proteins SMAD 1, 5 and 8 are substrates of type I receptors usually engaged by members of the BMP/GDF-subgroup, whereas SMAD 2 and 3 are typically activated by type I receptors triggered by TGF-βs, Activins and Nodal (Fig. 1A,B). Upon phosphorylation the R-SMAD proteins assemble with the so-called Co-SMAD 4 forming a heterotrimer required for nuclear translocation [73]. Two further SMAD proteins SMAD 6 and 7 (I-SMAD for inhibitory SMAD) can impede phosphorylation of R-SMADs hereby negatively regulating SMAD activation (Fig. 3D). All R-SMADs recognize the DNA-sequence CAGA but bind this sequence only with low affinity [74], additional motifs, usually targeted by partner co-factors, are required to obtain sufficient binding affinity for transcriptional regulation, but also to acquire cell-type specific selectivity for target genes (for review [6,75]). Thus the cell-type specific presence or absence of these SMAD co-factors or a coupling of the TGF-β SMAD pathway to other signaling pathways, which provide (or activate) the proper co-factors, can then enable transcriptional regulation of a cell-type specific set of target genes specific for a particular TGF-β member. Such coupling or cross-talk has been reported for several growth factor-signaling pathways, e.g. the Wnt/β-catenin, the LIF, or the TNF- pathway (for review [76]). Besides this cross-talk between different signaling factors also non-SMAD or SMAD-independent signaling pathways directly emanating from TGF-β receptors were postulated in particular involvement of MAP kinase pathways [77] (Fig. 3D). For instance, the regulatory subunit p85 of the phosphoinositide 3-kinase (PI3K) associates with the TGF-β receptor. The regulatory subunit p85 then activates PI3L in a ligand-dependent manner thereby linking the PI3K/Akt pathway to TGF-βs [78]. Similarly, BMP receptors can form complexes with the intracellular TGF-β-activated kinase 1 (TAK1) through the binding partners TAB1 and the ubiquitin E3 ligase XIAP, which is an inhibitor of apoptotic caspases. This leads to activation of the p38 kinase and shows that responses can be induced via SMAD-independent signaling pathways [79] (Fig. 3D). A recent review summarizes the results of ongoing research trying to identify additional components associating with the cytoplasmic domains of TGF-β receptors and known TGF-β signaling components [80]. The multitude of interaction partners already found so far suggests that the intracellular TGF-β signaling resembles more a large entangled signaling network rather than a linear cascade.

6. Many ligands – too few receptors

In contrast to the number of TGF-β ligands, which increased dramatically during evolution, the number of available type I and type II receptors seems amazingly low, particularly when assuming a mechanism such as one factor – one receptor – one function (Fig. 1A,B). In worm five potential ligands face two type I and one type II receptors, in fly the seven TGF-β members signal via three type I and two type II, but in humans only seven type I and five type II receptors have to suffice for the more than 30 TGF-β ligands [6,81]. This numeral discrepancy between the number of TGF-β ligands and available receptors immediately implies that a particular receptor of either subtype has to bind more than one ligand. In very few cases usage of a receptor seems confined to one TGF-β subfamily or even a single TGF-β ligand. Only the Anti-Muellerian Hormone (AMH) type II receptor (AMHR-II) is solely activated by AMH [82]. The TGF-β type II receptor (TβR-II) only binds to the three isoforms TGF-β1, -β2, and -β3 but no other TGF-β superfamily members (Fig. 1A). All other type I and type II receptors are shared among ligands of one subfamily or even are used by members from different subfamilies [83]. Among the type II receptors the Activin type II receptors ActR-II and ActR-IIB are most widely utilized (Fig. 1A). This is of special interest as ActR-II and ActR-IIB exhibit a dual signaling specificity [84]. By binding to members of the Activin subfamily (or GDF-8/GDF-11) the Activin type II receptors are involved in activation of the SMAD 2/3 pathway whereas binding to members of the BMP/GDF subfamily results in activation of SMAD 1/5/8. Some opposing activities of Activin-A and BMPs might be thus explained with competing binding to the Activin type II receptors [85]. Therefore one receptor can participate in different signaling complexes leading to distinct SMAD responses.

But we do not only see receptors binding various ligands from one or more TGF-β subfamilies, usually a particular TGF-β ligand can also bind to several TGF-β receptors of either subtype. This phenomenon called ligand–receptor promiscuity is particularly (but not exclusively) present in the BMP/GDF subfamily. In vitro

binding analyses show that the three type II receptors BMPR-II, ActR-II and ActR-IIB interact similarly with any member of the BMP-2/4, BMP-5/6/7 and the GDF-5/6/7 subgroup (Fig. 1A). Even binding of Activin-A to either Activin type II or the BMP type II receptor in vitro differs only by a factor of 10 in affinity [86]. Chemical crosslinking experiments, biophysical in vitro analyses or functional studies employing receptor overexpression in cells suggest that BMP/GDF ligands from the GDF-1/-3 [87], the BMP-3/GDF-10 [88] or the GDF-8/-11 [89] subgroup are more selective, binding specifically to the Activin type IIB receptor (ActR-IIB). Instead, several TGF-β ligands from different subfamilies such as members of the GDF-9/BMP-15 subgroup [90,91] or BMP-9 [92] exhibit strong preference for BMPR-II. But not only type II receptors are widely shared between different TGF-β ligands also type I receptors, which restrict the downstream SMAD pathway are jointly used (Fig. 1A). Members of the BMP-2/4, BMP-5/6/7 and GDF-5/6/7 subgroup and also AMH bind to the type I receptors BMPR-IA (ALK3) and BMPR-IB (ALK6) although with variable affinities [86]. The type I receptor ActR-I (ALK2) is also bound by members of the BMP-5/6/7 subgroup besides BMPR-IA and BMPR-IB. But in vitro interaction analyses reveal that its binding affinity to BMP-6 and BMP-7 is the lowest among the type I receptors recognized [86]. Surprisingly, cell-based experiments using deglycosylated BMP-6 specifically deficient in binding to ActR-I (ALK2) suggest that ActR-I is nevertheless essential as signaling type I receptor for the BMP-5/6/7 subgroup. Here, BMPR-IA cannot replace ActR-I (e.g. in C2C12 cells) despite exhibiting a 15 to 20fold higher in vitro binding affinity compared to ActR-I [93]. The Activin type I receptor ActR-IB (ALK4) is probably the most promiscuous type I receptor for members (BMP-3, GDF-1/3, Activin/Nodal, GDF-8/11) activating the SMAD 2/3 pathway [87,94–97]. For some ligands the co-receptor Cripto is required for binding to ActR-IB (ALK4) [87,96]. Other TGF-β superfamily ligands besides TGFβ1/2/3, i.e. GDF-8 or GDF-9, also bind the TGF-β type I receptor TβR-I (ALK5) showing that even this type I receptor is not limited to a particular ligand subfamily [97,98] (Fig. 1A). The TGF-β receptor ActR-IC (ALK7) has initially been identified as orphan receptor, but now serves as an alternative type I receptor for Nodal [96], furthermore certain Activin isoforms can also use ActR-IC [99]. The TGF-β superfamily receptor type I (TSR-I or ALK1) is a highly interesting member, as very few interacting ligands have been identified so far. Whereas BMP-9 has been shown to interact with TSR-1 with high affinity [92], TSR-I can also serve as an alternative type I receptor for TGF-β1/2/3 [100] (Fig. 1A). So far all ligand-type I receptor combinations were strictly restrained to a single SMAD pathway, either 1/5/8 or 2/3, even when a ligand exhibits promiscuous binding to various type I receptors. The usage of TSR-1 by TGF-β1/2/3 and the subsequent activation of the SMAD 1/5/8 pathways breaks this rule, showing that a TGF-β ligand depending on the cellular context can induce both otherwise strictly separated pathways (Fig. 1A). Although the above-described scenario immediately implies a highly promiscuous interaction network between different ligands and a limited set of receptors, one has to be cautious in interpreting the available data for the ligand–receptor promiscuity. Whereas biophysical interaction analyses such as surface plasmon resonance might provide for very accurate affinity data these measurements might not reflect the situation in organisms, as it needs to be combined with expression data to find in vivo examples where such combinations of promiscuous ligands and receptors are present at the same place and time. Similarly, ligand–receptor interaction analysis using chemical crosslinking data on cells also usually use recombinant factors as probes neglecting temporospatial expression of the binding partners analyzed and the crosslinking might also dependent on ligand/receptor reactivity. However, that the pronounced ligand–receptor promiscuity in the TGF-β superfamily might indeed affect TGF-β/BMP signaling also in vivo can be deduced from various studies analyzing the development processes. For instance studies of limb development [34] have shown that different BMP ligands with overlapping receptor usage act in a spatially and timely highly confined space and thus ligand–receptor promiscuity is likely to occur during this process.

7. A different assembly explains high ligand–receptor specificity for TGF-β1/2/3

This highly interweaved and complex ligand–receptor network raises two immediate questions. First, what is the molecular basis for this promiscuous ligand–receptor interaction considering sequence identity of sometimes less than 30% between receptor-sharing TGF-β ligands? Structure analyses have revealed a highly conserved butterfly-shaped dimer structure for the mature region of TGF-β ligands (Fig. 3A,B). The architecture resembles two hands (one for each monomer) assembled palm-to-palm, the β-sheets are described as fingers, the -helix and the dimer interface forming the palm, and the N-terminus resembling the thumb (for review [81]). Mutagenesis studies as well as structures of TGF-β ligand–receptor complexes clearly show that promiscuous receptor binding is not achieved by using different epitopes for each combination [101–105]. Activins and BMP/GDF ligand members all seem to bind their type II receptors via the so-called knuckle epitopes (back side of the fingers), whereas type I receptor binding occurs in the wrist epitope, which is formed by the front side of the fingers and the palm of the TGF-β ligand [106] (Figs. 3B, 4A,B). Only the TGF-β1/2/3 are exceptions, with the type II receptor TβR-II binding to their finger tips (Fig. 4D,E), differences in the affinities of the three TGF-β isoforms to TβR-II are due to amino acid differences in the fingertips [107]. Comparing the structures of the ternary complexes of TGF-β3:TβR-I:TβR-II and BMP-2:BMPR-IA:ActR-IIB shows that the high specificity for the TGF-βs – type II receptor interaction is due to differences in length and conformation of the finger tips in TGF-βs allowing only binding of TβR-II to TGF-βs but not to BMPs. Binding of TβR-II to other BMP/GDF ligands is impeded by the structural differences clearly separating TβR-II from the other type II receptors ActR-II, ActR-IIB and BMPR-II. Furthermore, several amino acid differences in the region equivalent to BMP-2's knuckle epitope prevent binding of the type II receptors ActR-II, ActR-IIB or BMPR-II to TGF-βs. Most evident, residue Ala34 in the knuckle epitope of BMP-2, which yields an antagonist upon A34D mutation [106], is occupied by a conserved glutamate residue in all three TGF-β isoforms. Also the position and orientation of the type I receptor TβR-I differs in the ternary complex TGF-β3:TβR-I:TβR-II from that found for the type I receptor in structures of BMP and GDF ligand–receptor complexes (Fig. 4A-E). The ectodomain of TβR-I is shifted towards the fingertips of TGF-β3 and the type I receptor binding is mediated by direct contacts between TβR-I and TβR-II (Fig. 4E,F). Such receptor–receptor contacts are absent in BMP ligand–receptor complexes explaining the non-cooperative binding for BMPs (Fig. 4C). Consequently, the highly cooperative binding of TβR-I in the TGF-β ligand–receptor complex formation requires the prior presence (or complex formation) of TGF-βs with TβR-II to allow type I receptor binding, which raises the question how TβR-I binds to GDF-8 or GDF-9, which associate with ActR-IIB via the classical knuckle epitope as seen for Activin-A [105]. Despite that structure analysis of GDF-8 bound to the modulator Follistatin [108] shows that the so-called pre-helix loop (see also Fig. 3B), an important element for type I receptor specificity in BMP ligand–receptor interactions [109–111], adopts a TGF-β like conformation and thus should enable TβR-I binding to GDF-8, the different position of type I and type II receptor will disrupt direct receptor–receptor interface essential for TGF-β receptor complex formation.

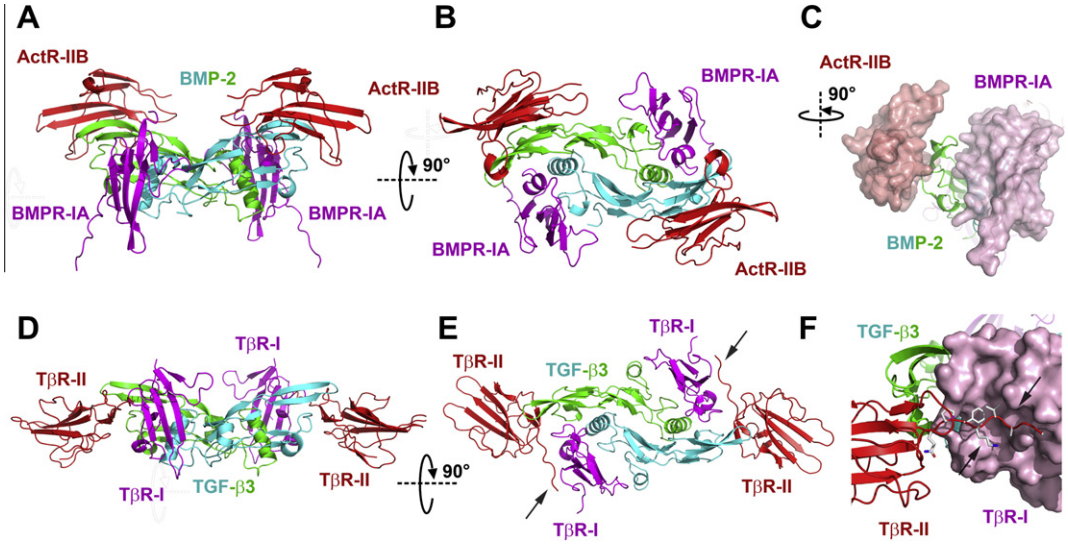

Fig. 4. (A) Structure of the ternary complex of BMP-2 bound to the extracellular domains of BMPR-IA and ActR-IIB. The two ActR-IIB receptors bind to the knuckle epitope of each monomer, whereas the two BMPR-IA receptors interact with the wrist epitopes. (B) As in A) but rotated around the x-axis by 90°. (C) In contrast to TGF-β3 ligand–receptor interaction, no direct contacts exist between the extracellular domains of the type I and type II receptors in BMP ligand–receptor complexes. (D) Structure of the ternary complex of TGF-β3 bound to the extracellular domains of TβR-I and TβR-II. (E) As in (D) but rotated around the x-axis by 90°. The type II receptor ectodomains interact with the tips of fingers 1 and 2 of each TGF-β3 monomer. The TβR-I receptor binds to the wrist epitope, but its orientation and location is slightly shifted compared to BMP ligand–receptor complexes. This results in direct contacts between the N-termini of the type I and type II receptors explaining the cooperative binding observed for the TGF-β ligand–receptor interaction (marked by arrows). (F) Cooperative binding of the TβR-I is due to a direct interaction between the TβR-I N-terminus and TβR-II (shown as magenta van-der-Waals surface).

8. Molecular basis for promiscuity and specificity

As the location of the receptor binding epitopes in Activin and BMP/GDF ligands are the same (Fig. 3A,B), other mechanisms such as different amino acid motifs must exist to ensure type I and type II receptor selectivity if required. In particular the type II receptors ActR-IIB and ActR-II are highly promiscuous and bind to many different Activin and BMP/GDF ligands. Several structures of complexes of different ligands (BMP-2, BMP-7 and Activin-A) bound to either ActR-II or ActR-IIB provide insight into promiscuity and specificity of the type II Activin receptors [101,102,105,112,113]. Mutagenesis has established the functional epitope of BMP-2 to BMPR-II and ActR-II showing that the type II receptor epitope is dominated by hydrophobic interactions [106], which has been confirmed also by the above-mentioned structural studies. However, of 24 residues of BMP-2 forming the interface mutagenesis has only found six to significantly influence type II receptor binding [106], with only one position (Leu90 in BMP-2) being absolutely conserved among all Activin type II receptor binding ligands (Fig. 5A).

This lack of a highly conserved interface between ligands and their shared receptors ActR-II and ActR-IIB might on the one hand be explained by the concept of so-called hot spot of binding introduced by James Wells in 1995 [114] (Fig. 5B,C). Herein, the residues in the center of a protein–protein interface contribute the majority of the overall binding energy of an interaction, whereas surrounding residues provide sealing from the environment. Thus the central residues are usually conserved, whereas peripheral amino acid residues can be exchanged as long as their sealing function is maintained (Fig. 5A–C). More than one hot spot can exist via modular interfaces and by combining different hot spots of binding a scalable affinity can be achieved [115]. The latter seems also to apply for the Activin type II receptor interactions. Whereas BMP-2 (and other BMPs and GDFs from the BMP-5/6/7 and GDF-5/6/7 subgroup) binds ActR-II and ActR-IIB with rather low micromolar affinities, binding of these receptors by Activins occurs with very high affinity in the nanomolar range [86]. There is evidence (although pull down experiments provide only semi-quantitative data) that all TGF-β ligands binding to SMAD 2/3 activating type I receptors seem to exhibit high affinities for their type II receptor(s), and BMPs and GDFs activating the SMAD 1/5/8 pathway bind rather their type I receptors with high affinities. Whether this is a general mechanism that possibly also influences signal transduction and further discriminates the SMAD 1/5/8 and SMAD 2/3 pathways remains to be resolved by further quantitative functional analyses.

The search for the type II receptor affinity difference between BMPs and Activins revealed that indeed a second hot spot of binding exists in the ligand-type II receptor interface. Sequence and structure comparisons of the Activin-A:ActR-II and the BMP-2:ActR-IIB interactions showed that only two residues differing between BMP2 and Activin-A can switch a low-affinity to a high-affinity epitope [101] (Fig. 5D,E). Mutation of Leu100 and Asn102 in BMP-2 to the equivalent amino acids Lys and Asp in Activin-A enhances the affinity by shielding a hydrogen bond between Ser88 of BMP-2 and a backbone amide in the ActR-IIB receptor from the environment (Fig. 5D,E). Thus a high-affinity epitope to ActR-IIB might consist of the general hydrophobic hot spot of binding (which is centered around Ala34 and Leu90 in BMP-2) used by all Activin and BMP type II receptors [101,116]. It is remarkable that the "shielding" residues (equivalent to Leu100 and Asn102 in BMP-2) show indeed little variation with respect to their side chain size showing larger side chains at the first and second position for most ligands with assumed high affinity for ActR-IIB. As the affinity differences mediated by the exchanges at these two positions were quite specific for ActR-IIB and did not affect interaction of ActR-II (nor BMPR-II) with BMP-2 (Fig. 5D,E), specificities for the type II receptors might be achieved through the use of distinct additional hot spots. Further mutations in BMP-2 provided two other positions specifically modulating the affinity for BMPR-II [101], and a structure-/function study showed that the high ActR-IIB specificity of BMP-3 is due to a charge–charge pair involving a lysine residue to BMP-3 (Lys30) and a glutamate in ActR-IIB (Glu76), which is a lysine in ActR-II [88]. Interestingly,

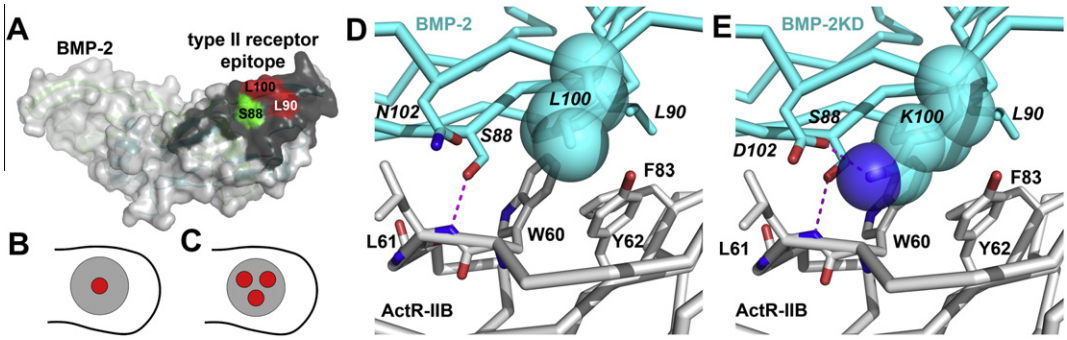

Fig. 5. (A) The hotspot of binding in BMP-2 for the BMP type II receptor interaction consists of a hydrophobic patch formed by two residues (Leu90 and Leu100, marked in red) located in the center of the large type II receptor contact area (marked in dark gray). A nearby second hot spot of binding mediated by a hydrogen bond (shown in green) is activated in the high-affinity Activin-A type II receptor interaction. (B) A hot spot of binding usually consists of a central bond (marked in red), which is either a polar (hydrogen bond) or hydrophobic interaction, which is then shielded from the environment by a shell of complementary (hydrophobic or polar, respectively) residues. (C) Shared interaction components that interact with their different binding partners with variable affinities often contain several hot spot of bindings, some of which are used for low affinity binding and all of them together can enforce high affinity binding. (D) Low-affinity binding of BMP ligands to the Activin type IIB receptor only utilizes the hydrophobic patch. (E) "Activation" of the second hotspot by mutating two "shielding" residues (L100K, N102D) in close proximity confers high affinity binding of BMP-2 (BMP-2KD) to ActR-IIB. The additional binding affinity is not due to direct ligand–receptor interactions via the two mutated residues, but due to protection of the hydrogen bond between the hydroxyl group of the conserved serine (Ser88 in BMP-2) and a main chain amide of the type II receptor (Leu61). The amino acid replacements block water from accessing the central hydrogen bond thereby increasing its contribution to the binding energy.

the lysine residue is conserved between several TGF-β superfamily ligands, e.g. Nodal, GDF-8, GDF-11, which are reported to use ActR-IIB as their high affinity receptor. The hot spot of binding mechanism is not limited to the ligand-type II receptor interface but is also found in the BMP-2 interaction with their high affinity type I receptors BMPR-IA (ALK3) and BMPR-IB (ALK6). Alanine scanning mutagenesis did first not reveal residues altering binding affinity by more than factor of 10 [106]. A refined structure-/function analysis then revealed that a conserved hydrogen bond pair in the BMP type I receptor interface is the hot spot and that the surrounding residues mainly shield this polar interaction from destabilizing access of solvent [109].

The concept of hot spots of binding together with the likely combinatorial use of a modular interface with several potential hot spots possibly forms the basis of promiscuous type II receptor binding by Activin and BMP/GDF ligands with the concurrent capability to scale affinity by more than 50fold (Fig. 5B,C). This implementation possibly also explains the structural variability of the plethora of extracellular antagonist, modulator proteins, or co-receptors identified for the ligands of the TGF-β superfamily (Fig. 6A–H). Structures of several of these modulator protein–TGF-β ligand complexes have been determined in the past, e.g. BMP-7:Noggin (Fig. 6B,F) [117], Follistatin bound to Activin or GDF-8 (Fig. 6C,G) [108,118,119], or of the Chordin-related first Von Willebrand type C (VWC1) domain of Crossveinless-2 (CV2) bound to BMP-2 (Fig. 6D,H) [120]. None of the three examples of modulator proteins share any structural similarity; nevertheless all bind to the same epitopes also used by type I and type II receptors of the TGF-β superfamily (Fig. 6). Functional studies employing mutagenesis show that even though the Von Willebrand domain and the BMP type II receptors are structurally completely distinct, the same hot spots, e.g. Leu100 in BMP-2, are used for binding [120]. Thus, the need of only a few conserved residue pairings with a (large) group of variable surrounding residues to shield and maintain the core interaction will allow the development of structurally highly different interaction partners.

9. Structural flexibility and adaptability

Besides the hot spot of binding mechanism inherent structural flexibility of TGF-β ligands and their receptors has been identified as a possible source to modulate ligand–receptor specificity. Structure analyses of "free" type I and type II receptors have indicated that the receptor ectodomain can perform an induced fit mechanism of variable degree upon binding to the ligand (Fig. 7A–C). For the type II receptors crystal structures of ActR-II and BMPR-II in their unbound form reveal that the core ligand-binding interface is preformed and only peripheral loops undergo conformational rearrangement upon complex formation [121,122]. In contrast, NMR structure analysis of the BMP type I receptor BMPR-IA showed that the majority of its ligand-binding interface is unstructured and highly dynamic [123]. Upon binding to BMP-2 a 15mer loop passes through a disorder-to-order transition folding into a highly defined segment with a 1.5-turn helix in the center (Fig. 7A,B). Most interestingly, this segment holds the hot spot of binding of BMPR-IA for recognizing and binding to BMP-2 indicating that a fixation or pre-orientation of the residue(s) serving as hot spot of binding is not required. That the flexibility of the loop segment in BMPR-IA can really serve to adapt to different geometries and thus might potentially enhance binding partner diversity was confirmed from structure analysis of the BMPR-IA ectodomain bound to the neutralizing antibody raised against BMPR-IA (Fig. 7C) [124]. Here, the antibody as well as the physiological ligand BMP-2 bind to the very same epitope of the common partner BMPR-IA but induce vastly different conformations in the flexible segment. Thus the flexibility seen in the ligand binding epitope of BMPR-IA might be utilized for interface adaptation during binding of different ligands although the structural similarity of BMPR-IA-recognizing BMP ligands will require a much smaller conformational adaptation compared to the antibody. A flexible recognition segment is not a common motif in all type I receptors of the TGF-β superfamily, as structure analysis of TβR-I shows that here the equivalent region is structured and rigid by introducing proline residues and the pre-ordered structure is essential for TGF-β binding and specificity [125]. Another example in which flexibility is used to create specificity is the type I receptor specificity of GDF-5 (Fig. 7D,E). Studies of knockout mice suggest that GDF-5 signaling through the BMP type I receptor BMPR-IB (ALK6) is important for correct GDF-5 function [34,126]. Although chemical crosslinking of GDF-5 could only be detected to BMPR-IB suggesting an exclusive interaction [127], in vitro binding analyses show that GDF-5 binds to BMPR-IB with only a 10 to 15-fold higher affinity than to BMPR-IA [86,110]. A single residue could be identified in GDF-5, Arg57 located in the wrist (type I receptor binding) epitope,

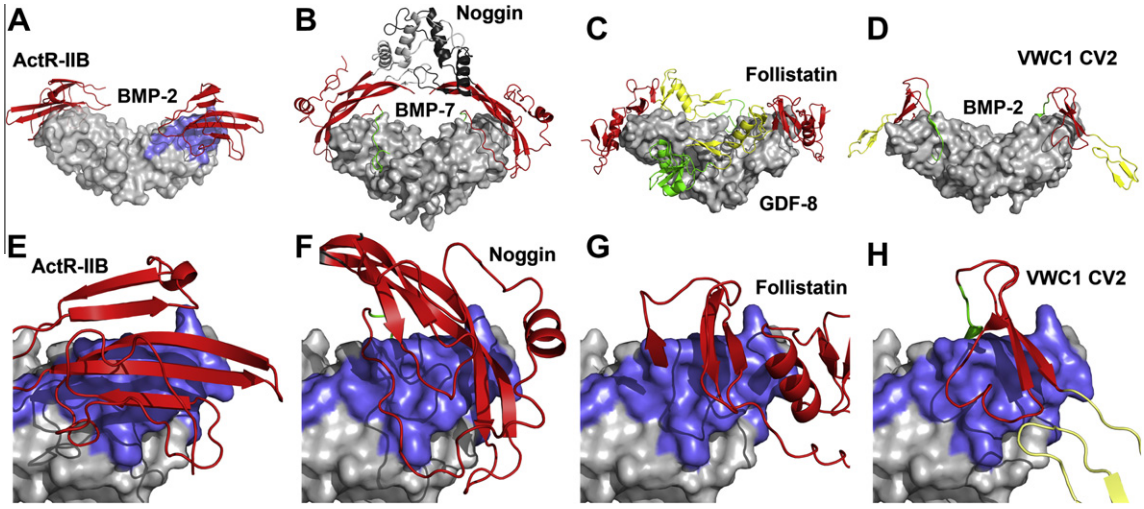

Fig. 6. (A–D) The concept of hot spot of binding, which requires little sequence conservation in large protein–protein epitopes possibly explains the structural variability of knuckle–epitope interacting binding partners of BMP/GDF ligands. A comparison of the complexes of BMP-2 – ActR-IIB **(A)**, BMP-7:Noggin **(B)**, GDF-8:Follistatin **(C)** and BMP-2:Van Willebrand domain **(D)** (BMP-2:VWC1 of Crossveinless 2) reveals no structural similarities between the different BMP binding partners, which nevertheless bind to the same epitope of the BMP/GDF ligands. Red regions interact with the type II receptor epitope, green regions interact with the type I receptor epitope, yellow regions either do not contribute to binding or constitute additional linker domains. (E–H) Close-up of the interactions between the BMP knuckle epitope (marked in purple) and the receptor (E, ActR-IIB) or the modulator proteins (Noggin (E), Follistatin (F), or the Von Willebrand domain 1 of the BMP modulator protein Crossveinless 2 (G)). All of these binding partners show the overlapping (promiscuous) binding to various ligands of the BMP and GDF subgroup.

which "encodes" for this type I receptor specificity [110]. Mutation of this residue in GDF-5 to alanine, the equivalent amino acid in BMP-2, leads to a complete loss of discrimination between BMPR-IA and BMPR-IB and results in a GDF-5 variant R57A, which exhibits BMP-2 like binding characteristics [110]. A similar mutation R57L is also found in patients suffering from the skeletal malformation disease symphalangism and shows that type I receptor specificity is essential for proper GDF-5 signaling [128]. Structure analysis of the GDF-5:BMPR-IB complex now show that a receptor loop segment, which contacts Arg57 in GDF-5 exists in two different conformations in BMPR-IB and BMPR-IA (Fig. 7D,E) [111]. Whereas in BMPR-IB the loop adopts a conformation providing sufficient space for the bulky arginine side chain, the loop conformation found in BMPR-IA would result in steric hindrance, thereby explaining the lower binding affinity of GDF-5 to BMPR-IA (Fig. 7E indicated by a red circle). Interestingly, the specificity-determining bulky residue arginine is conserved in all BMPR-IB specific TGF-β ligands, i.e. GDF-5, -6, -7 and BMP-15.

Another form of possibly creating type I receptor specificity is seen for TGF-β2 and Activin-A. Structures of the binary complexes bound to their type II receptors TβR-II and ActR-II present TGF-β ligands deviating from the canonical butterfly-shaped dimer architecture (Fig. 7F–H) [113,129]. The dramatic opening of the TGF-β3 dimer (Fig. 7F) but also the wing beat motion of Activin-A (Fig. 7G,H) completely disrupt the type I receptor epitope, but the canonical dimer architecture would be formed upon "fixing" the ligand via binding to two type II receptors on the cell surface [113]. NMR relaxation studies show that the dimer interface of free TGF-β3 is indeed destabilized and would thus be consistent with such a limited unfolding of the ligand dimer [130]. One might argue that this change in dimer architecture might be important for type I receptor specificity as the disrupted type I receptor epitope is unable to bind BMP type I receptors such BMPR-IA, BMPR-IB or ActR-I (ALK2). However, once the canonical butterfly dimer architecture is formed other mechanism must ensure discrimination between the different type I receptors and thus this reasoning seems unlikely. One other explanation might be the establishment of a stepwise sequence of receptor binding such that first type II receptor binding occurs through the unaffected epitopes leading to subsequent formation of the type I receptor epitopes by constraining the ligand flexibility and allowing type I receptor binding only as the second step.

10. Ligand–receptor promiscuity versus functional specificity

Despite our knowledge of how promiscuity and specificity can be generated, one important question resulting from the pronounced promiscuity in TGF-β ligand–receptor interaction remains unanswered: How is functional diversity and specificity of TGF-β signaling achieved? Although TGF-β signaling likely uses "co-signals" from other morphogens such as Wnt or other cytokines (see above), which by applying double morphogen gradients precisely determine cell differentiation and fate in a temporospatially defined manner, it cannot explain whether or not two TGF-β ligands will deliver the same qualitative and quantitative signal output when using the same receptors. Such a situation would exist in vivo during limb and joint development, where many different TGF-β ligands act in a highly overlapping cellular space and their overlapping receptor usage will inevitably lead to signaling interference (for review [131]). For instance, BMP-2 and GDF-5 (but also other BMP factors) show expression patterns highly overlapping in time and space in the developing limb and due to their promiscuous receptor binding can form receptor assemblies with identical composition. What is our expectation in such a case? If only composition defines signal outcome both ligands should encode the same function and we would thus observe redundancy. However, deletion studies of *Gdf5* suggest that signaling of GDF-5 is different from BMP-2 [34]. One could argue that the signaling of GDF-5 specifically through BMPR-IB might cause the different signaling and reports indeed show that BMPR-IA and BMPR-IB can exert opposing effects [132]. In vitro cellular assays in two different cell lines routinely used in BMP analysis, C2C12 and ATDC5, allowed studying differences in signaling in more detail and a defined background. As both cell lines C2C12 and ATDC5 seem not to express the type I receptor or express BMPR-IB to a very low extent [133,134], it is very likely that signaling of both ligands, BMP-2 and GDF-5, has to occur via the receptor BMPR-IA. Interestingly, in C2C12 cells only BMP-2 but not GDF-5 can induce expression

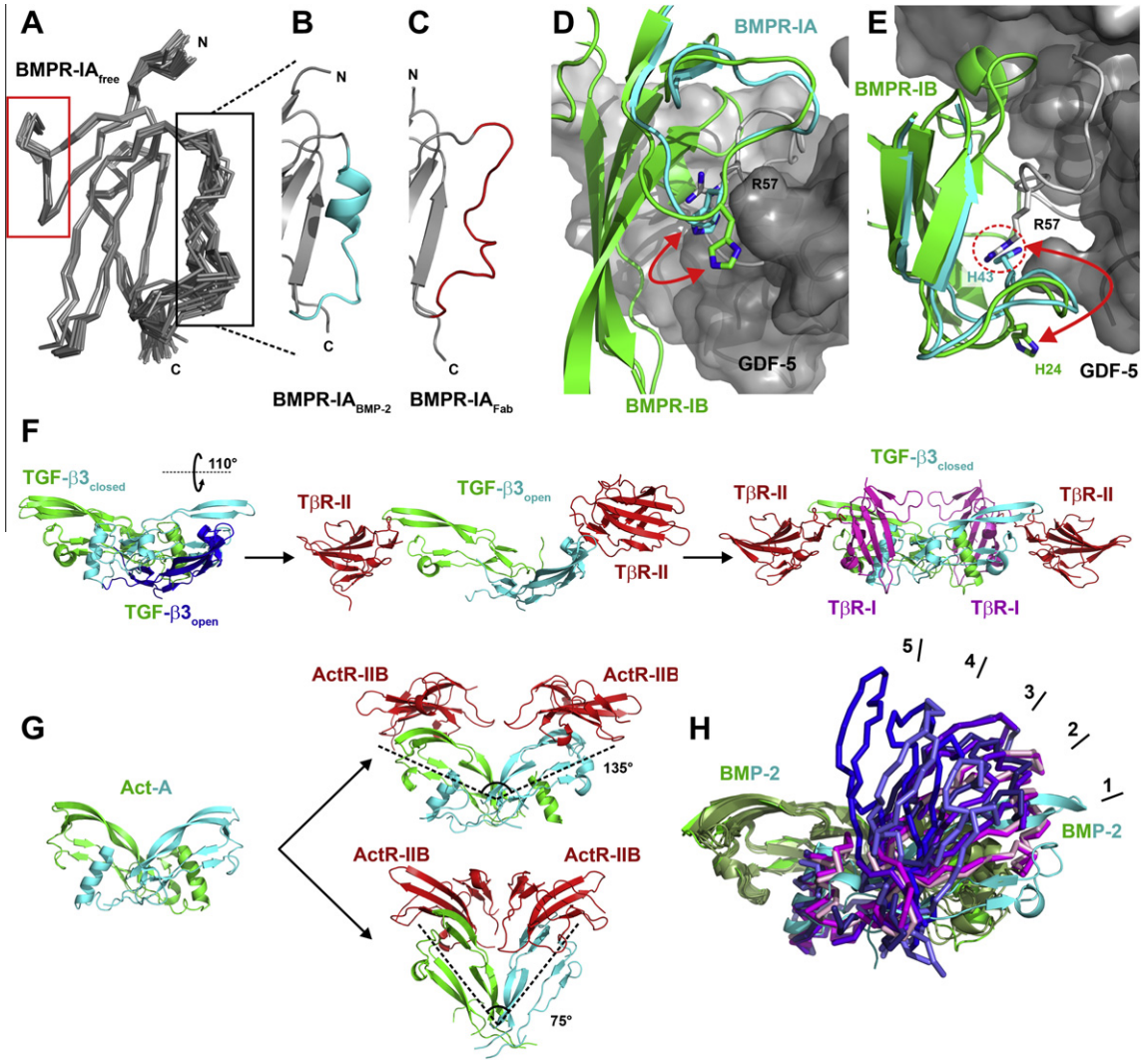

Fig. 7. (A) NMR analysis of the free ligand-binding domain of BMPR-IA reveals a disordered and dynamic structure for the β4β5-loop, which constitutes a large part of the binding epitope to BMP-2 (black box). (B) Upon binding to BMP-2 the flexible element (marked in cyan) becomes structured and adopts a defined structure comprising a short 1.5-turn helix. (C) The dynamic nature allows to adapt the loop conformation according to the needs of the binding partner as seen from the extended, but rigid conformation of the loop element (marked in red) when bound to a neutralizing antibody. (D) A second loop element (marked by a red box in (A)) of the BMP type I receptors can exist in two different conformations. In BMPR-IB (green) the β1β2-loop adopts an open conformation allowing the binding of BMP/GDF ligands with a large bulky residue (Arg57 in GDF-5) in the ligand's pre-helix loop. In BMPR-IA this loop is in a closed conformation resulting in a steric hindrance between a large side chain at position 57 and residues, e.g. His43 in BMPR-IA (equivalent residues in BMPR-IB His23). Thus high-affinity binding of BMPR-IA is only possible for ligands with small amino acids at the position equivalent to Arg57 in GDF-5. BMPR-IB does not discriminate ligands with differing amino acid side chains at this position. (E) As in (D) but rotated around the x-axis by 90°. The conformational change is indicated by arrows. (F) TGF-β3 can exist in two conformations (differing by a rotation of 110° perpendicular to the intermolecular disulfide bond) as seen from structure analysis of free TGF-β3 and TGF-β3 in complex with TβR-II (middle panel). For ternary complex formation a closed conformation is required for binding of the type I receptor TβR-I (right panel). The switch between an open and closed ligand conformation might ensure a sequential binding mode and possibly increases type I receptor specificity. (G) A flexible dimer architecture is also seen for Activin-A. Structure analyses of free Activin-A and Activin-A bound to its type II receptor ActR-IIB have revealed a wing flap motion with a highly variable interdomain angle. (H) Superposition of the structures of BMP-2 (green and cyan) with different structures of Activin-A in unbound conformation and in conformations bound to either ActR-IIB or follistatin indicating the high variability in dimer architecture. The different interdomain orientations are indicated by numbers: 1 = BMP-2; 2 = Activin bound to Follistatin (PDB entries 2P6A and 2B0U); 3 = free Activin and Activin bound to ActR-IIB (PDB entries 2ARV and 1S4Y); 4 = Activin bound to a fragment of Follistatin (PDB entry 2ARP); 5 = Activin bound to ActR-IIB (PDB entry 1NYS).

of the osteogenic marker gene *Alp* (encoding for alkaline phosphatase), whereas in ATDC5 cells both ligands do induce alkaline phosphatase (ALP) expression. Here, a tenfold higher concentration of GDF-5 is required, which nicely correlates with the lower affinity for the BMPR-IA receptor [110]. To rule out that type I receptor affinity is responsible for the cell-specific differences in BMP-2 and GDF-5 mediated ALP expression, we used the GDF-5 variant R57A, which exhibits the same binding affinities for BMPR-IA and thus can be considered a BMP-2 mimic (Klammert, U. et al. manuscript in preparation). Despite its ability to form the same receptor assembly with the same efficiency as BMP-2, GDF-5R57A did not induce ALP expression in C2C12 cells. That GDF-5R57A indeed binds to the same receptors as BMP-2 could be confirmed in a competition experiment showing that the variant inhibits BMP-2 activity in a dose-dependent manner (Klammert, U. et al. manuscript in preparation). This indicates that the formation of a BMP receptor complex of identical composition can be active for one particular ligand, but silent for another suggesting that different TGF-β ligands might encode different intracellular signals despite using identical receptor assemblies. Thus ligand–receptor promiscuity is not automatically a direct evidence for functional redundancy.

So what are possible mechanisms that allow for a ligand-specific activation of otherwise identical receptor assemblies? One possibility might be small ligand-specific structural differences in

the complex architecture. Upon comparing the structures of different BMP/GDF ligand–receptor complexes, we have found small differences in the orientation of the type I receptors that, if translated into the cytoplasm, might influence not only transactivation between receptors but also phosphorylation of substrates and hence the downstream signaling cascade [101,111]. That signal transduction of single-span transmembrane receptors is not limited to receptor oligomerization by just lateral movements in the membrane, but might be modulated by vertical or rotational movements as has already been demonstrated in several studies [135,136]. The kinetics of the ligand–receptor interaction might be an important factor for the specificity of downstream signals. As the cytoplasmic receptor domains are kinases their substrate affinities and phosphorylation rate can be used to encode ligand-specific signals, if the ligand–receptor complexes differ with respect to their assembly lifetimes. We indeed have hints that the kinetics and not only thermodynamics of the ligand–receptor assembly might matter. Variants of GDF-5 exhibiting identical affinities for the BMP type I receptors as wildtype GDF-5, but differing in their binding kinetics do not activate gene expression in cellular assays [111]. Thus triggering the same receptor assembly but with a shorter lifetime might not activate downstream signaling or restrain the set of signals. A third possibility might be the involvement of co-receptors such as Cripto, endoglin, betaglycan and others having their own ligand specificity profile (see also Fig. 3D). If having a cytoplasmic domain these could directly alter signaling outcome. However also co-receptors without a cytoplasmic domain such as Cripto or the recently identified BMP-specific co-receptors of the RGM family (for review [137]) can modulate the signaling cascade in a ligand-specific manner by altering the receptor binding specificity of individual TGF-β ligands. Thus a large toolbox to enhance signaling diversity through a limited set of receptors might exist and requires our future attention.

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